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Effects of Lead on Benzene Metabolism

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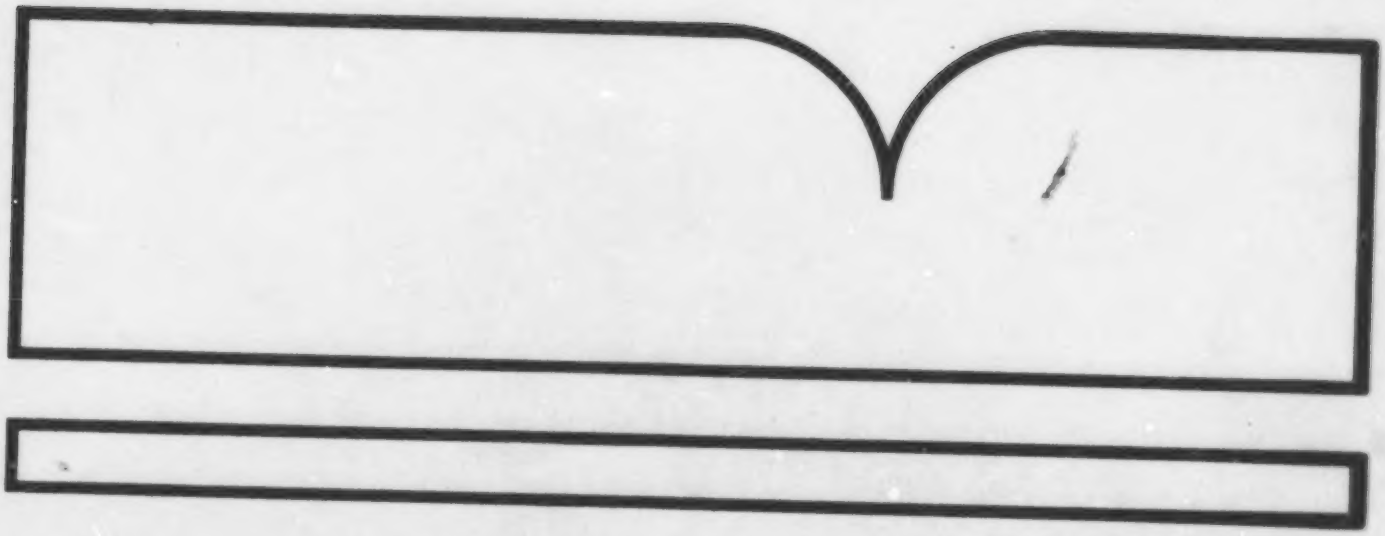

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16. ABSTRACT

The possibility that Pb could affect benzene metabolism, through inhibition of enzyme snythesis, was examined by studies of the effects of chronic oral Pb treatments on benzene conversion to phenol. Rats were given either distilled deionized water or 0.05, 0.58, 17 or 352 ppm Pb solutions as drinking water. After 6, 9, 12, and 15 wk of treatment, rats from each group were sacrificed and in vitro benzene metabolism by benzene hydroxylase was measured in liver enzyme preparations. After 24 wk, the remaining animals were injected ip with 400 mg/kg benzene and urinary phenol levels measured daily for 4 d.

The enzyme activity, studied in vitro, was significantly increased in animals that ingested the 352 ppm solution for 6 wk. Also, Pb levels in all groups of animals were significantly higher in tissues from animals that ingested 352 ppm Pb. Although urinary phenol levels accounted for 30-40% of the ip benzene dose, Pb ingestion had no significant effect on phenol excretion. These results suggested that oral Pb treatments had a significant effect on the enzyme responsible for benzene hydroxylation to phenol. However, unknown factors appear to compensate for these changes in vivo.

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EFFECTS OF LEAD ON BENZENE METABOLISM

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The possibility that Pb could affect benzene metabolism, through inhibition of enzyme synthesis, was examined by studies of the effects of chronic oral Pb treatments on benzene conversion to phenol. Rats were given either distilled deionized water or 0.05, 0.58, 17, or 352 ppm Pb solutions as drinking water. After 6, 9, 12, and 15 wk of treatment, rats from each group were sacrificed and in vitro benzene metabolism by benzene hydroxylase was measured in liver enzyme preparations. After 24 wk, the remaining animals were injected ip with 400 mg/kg benzene and urinary phenol levels measured daily for 4 d.

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INTRODUCTION

Petroleum workers and service station attendants are exposed to a combination of Pb and benzene, two additives in gasoline fuel. Benzene is metabolized by the cytochrome P-450 mixed-function oxidase (MFO) enzyme system of liver, lung, and kidney tissues. Evidence suggests that although metabolism facilitates excretion of benzene, it is also responsible for toxic effects observed after benzene exposure (Hutson, 1970; Lee et al., 1974; Andrews et al., 1975, 1977; Snyder et al., 1977). Pb could interfere with benzene metabolism since acute doses can reduce MFO activity through inhibition of heme synthesis and cytochrome P-450 content (Alvares et al., 1972; Scoppa et al., 1973). Therefore, during an occupational exposure to gasoline fuel, the presence of Pb could change the metabolism of benzene in workers.

Two studies were conducted to elucidate the effects of Pb ingestion on benzene metabolism to phenol. The purpose of the first study was to

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determine whether metabolism of benzene by the MFO enzyme system was altered in rats that ingested $PbCl_2$ solutions. To determine whether Pb affected *in vivo* benzene metabolism, a second study was conducted. Rats were treated for 23 wk with $PbCl_2$ and the metabolism of a single ip benzene injection was determined by measuring urinary excretion of phenol. In both investigations, the body Pb burden was measured by analysis of Pb concentrations in femur, liver, and blood tissues.

EXPERIMENTAL PROCEDURES

Animals

Rats were housed in 20 X 51 mm raised cages with food (Wayne Lab-Blox) and drinking water solutions continuously available. They were kept on a $10\frac{1}{2}$ h on–$13\frac{1}{2}$ h off light cycle with room temperature 22°C and relative humidity 38%. Standard laboratory care practices were observed in accordance with the Regulation of the American Physiological Society for the Care and Use of Animals.

Forty-five Wistar strain female rats (Charles River Breeding Laboratories, Wilmington, Mass.), 4 mo old (250–300 g), were separated into one control group of 5 animals and four treatment groups of 10 animals each. The control group received deionized-distilled drinking water and the treatment groups received drinking water solutions of 0.05, 0.58, 17, or 352 ppm $PbCl_2$ in deionized-distilled water. Pb doses were calculated as 16% of Pb intake, since 3-mo-old rats absorb this amount of Pb from the gastrointestinal tract (Kostial et al., 1971).

In the first study, animals were sacrificed after 6, 9, 12, and 15 wk of treatment. Two rats from each treatment group and one rat from the control group were killed, a total of nine animals, at each sampling period. Each liver was removed and minced, and 3–5 g tissue was stored at −20°C for Pb analysis. Both femurs and 5 ml whole blood from each animal were collected, stored at −20°C, and wet ashed for Pb analysis. The Pb concentration was measured by standard flame and furnace atomic absorption techniques (Gorsuch, 1970; Abu-Samra et al., 1975).

Benzene metabolism to phenol by benzene hydroxylase (E.C. 1.14.12.3, benzene 1,2-dioxygenase) was measured as previously described (Snyder et al., 1967; Gonasun et al., 1973; Harper et al., 1975). Liver homogenates (20%) were prepared in 0.1 *M* KCl, 0.1 *M* phosphate buffer and then centrifuged for 10 min at 4°C and 9000 rpm. The supernatants were diluted with buffer to a protein concentration of 2 mg/ml. The NADPH generating system added to 5 ml of each supernatant contained the following reagents: 1.2 μM NADP, 23 μM isocitrate, 2.5 μM $MgSO_4$, and 100 μM nicotinamide. Triplicate samples of supernatant from each liver were incubated with 10 μl benzene for 1 h at 37°C in a shaker-equipped water bath. Protein in the supernatants was determined by standard methods (Lowry et al., 1951).

After incubation of the samples with benzene, free phenol was extracted with isopropyl ether and detected with a gas chromatograph equipped with a flame ionization detector. A column packing of 25% polyethylene glycol succinate on acid-washed Chromasorb W was used to separate the phenol. Operating conditions were: column temperature, 185°C; detector and inlet temperature, 300°C; carrier gas, He; carrier gas flow rate, 30 ml/min. Unknown phenol was analyzed by comparison of the sample peak area with a standard peak area.

In the second study, the *in vivo* metabolism of benzene was determined in nine rats chronically dosed with $PbCl_2$ in drinking water at levels given above. The dose levels of benzene and general sampling protocol were identical to those used in a previous investigation (Cornish and Ryan, 1965). After 24 wk of Pb treatment, all animals were placed in rodent metabolism cages and urine samples were collected daily from each rat for the first 3 d. On d 4 all rats were injected ip with 400 mg/kg benzene, and urine samples were collected daily for the next 5 d.

Free, total, and conjugated urinary phenol levels were measured for each day. Free phenol was extracted as described above. Total phenol was measured by adding sulfuric acid to the remaining volume of each urine sample, which was then placed in boiling water for 1 h (Ikeda, 1964). Samples were cooled and free phenol was extracted with 15 ml isopropyl ether. Phenol samples were analyzed by the gas chromatography method previously described. Conjugated phenol was calculated as the difference between free and total phenol.

Data were evaluated statistically by using two-factor analysis of variance tests. The factors were time and treatment. Differences were considered significant at $p < 0.01$. Enzyme activity data were expressed in logarithmic form and corrected for an increase in control activity with time. Tissue Pb concentrations were statistically analyzed by using dry-weight values.

RESULTS

Liver Enzyme Changes

Enzyme activities of liver samples during 15 wk of Pb treatment are given in Table 1. Enzyme activity was highest in the 352 ppm Pb treatment group after 6 wk of treatment. A significant difference ($p < 0.01$) in enzyme activity was found with treatment dose but not with time. The equation that expressed this relation was:

$$\log \text{activity} = W_i - 0.03(T - \bar{T}) + 0.01(T^2 - \bar{T}^2)$$

where W_i is the mean activity for each week; T is the treatment dose; $\bar{T}$ is the mean of each treatment dose; and $\bar{T}^2$ is the mean for each treatment dose squared.

TABLE 1. Liver Benzene Hydroxylase Activity after Chronic Pb Ingestion

Pb treatment (μg/ml)	Pb dose, 6–15 wk (mg)	Enzyme activity[a]			
		6 wk	9 wk	12 wk	15 wk
0.00	0	99.2 ± 11.4[b]	98.2 ± 12.0	103.8 ± 1.0	120.0 ± 2.6
0.05	0.1–0.2	98.2 ± 6.8	84.5 ± 13.4	117.1 ± 16.9	91.7 ± 27.0
0.58	0.9–2.1	92.6 ± 20.1	109.6 ± 12.0	92.7 ± 21.5	126.4 ± 34.0
17.00	27.0–55.0	140.7 ± 23.6	116.4 ± 10.5	91.1 ± 4.8	96.4 ± 17.1
352.00	473.0–1251.0	150.6 ± 19.7	101.4 ± 10.9	114.5 ± 18.4	117.4 ± 25.5

[a] Activity is expressed as picomoles of phenol formed per minute per milligram of protein.
[b] Values are means ± SD of triplicate samples.

Urinary Phenol Cages

Changes in urinary phenol levels during the 7-d study are compared in Table 2 and Fig. 1. The amounts of free, conjugated, and total phenol excreted per day for all rats during the preliminary 3 d were 4.2 ± 4.0 μg free phenol and 0.4 ± 0.2 mg conjugated and total phenol. After injection of each rat with 400 mg/kg benzene on d 4, 26% was excreted as phenol

TABLE 2. Urinary Free and Conjugated Phenol from Animals Dosed with Pb and Benzene[a]

Pb treatment (μg/ml)	Week 24				
	1–3 d	4 d	5 d	6 d	7 d
			Free phenol (μg)[b]		
0.00	5.3 ± 3.2	13.2	2.2	1.5	0.6
0.05	5.1 ± 5.2	23.2 ± 21.1	2.9 ± 0.8	1.1 ± 0.2	1.5 ± 0.9
0.58	2.1 ± 1.5	15.3 ± 9.7	2.1 ± 0.7	0.8 ± 0.0	4.0 ± 0.5
17.00	6.2 ± 5.3	7.4 ± 0.8	0.6 ± 0.3	1.5 ± 1.2	0.9 ± 0.3
352.00	2.7 ± 2.6	56.9 ± 21.0	2.3 ± 2.0	1.0 ± 0.1	2.4 ± 2.8
			Conjugated phenol (mg)[b]		
0.00	0.5 ± 0.1	23.2	14.4	0.41	0.43
0.05	0.5 ± 0.3	33.3 ± 16.1	1.2 ± 0.6	0.4 ± 0.4	0.2 ± 0.1
0.58	0.3 ± 0.2	28.7 ± 4.9	18.2 ± 10.8	0.4 ± 0.2	0.6 ± 0.2
17.00	0.3 ± 0.1	39.9 ± 4.1	17.8 ± 5.0	0.3 ± 0.0	0.3 ± 0.1
352.00	0.3 ± 0.2	23.6 ± 2.4	11.1 ± 12.2	0.2 ± 0.0	0.2 ± 0.2

[a] Animals were dosed with lead chloride for 23 wk. Days 1–3 of wk 24 provided control urinary phenol levels before benzene injection. All animals were then injected with a single dose of 400 mg/kg benzene and urinary phenol levels were measured for the following 4 d. The Pb dose levels for 24 wk were calculated as: 0.05 and 0.58 μg/ml groups, less than 1 mg; 17 μg/ml group, 16 mg; and 352 μg/ml group, 342 mg.
[b] Values are means ± SE.

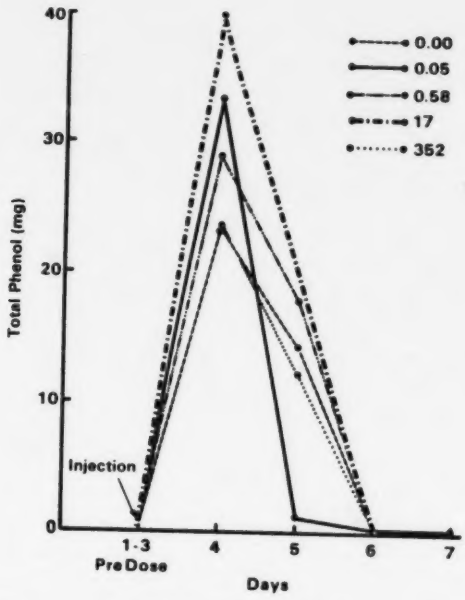

FIGURE 1. Total phenol excretion in animals after oral Pb treatment and ip benzene administration. After 23 wk of treatment with lead chloride, control total phenol levels were measured for d 1–3 of wk 24. Then all animals were injected with a single dose of 400 mg/kg benzene and phenol excretion measured for the next 4 d.

in the first 24 h. By d 5, urinary free phenol had returned to control levels. Conjugated and total phenol were 300% above control levels on d 5 but returned to baseline levels on d 6 and 7. By the end of the study 36% of the benzene dose had been recovered. Statistical analysis of the data showed that prior Pb treatment had no effect on the amount of phenol excreted after the benzene injection.

Tissue Lead Concentrations

A significant increase in femur Pb concentration in 24 wk occurred only in animals in the 352 ppm group ($p < 0.01$) and there was no effect of time on Pb accumulation. The femur Pb level in all animals, except those in the 352 ppm group, was 29.0 ± 6.2 μg/g.

Similarly, a significant accumulation of Pb in the liver occurred only in animals in the 352 ppm group ($p < 0.01$). No effect of time was observed. The liver Pb concentration for animals in the control, 0.05, 0.58, and 17 ppm groups was 1.2 ± 0.4 ppm and for animals in the 352 ppm group was 2.5 ± 0.2 μg/g. Pb was detected in blood samples only for the 352 ppm Pb group. After 9, 12, 15, and 24 wk of Pb treatment, blood Pb concentrations ranged from 0.1 to 0.6 μg/g.

DISCUSSION

Lead has been shown to inhibit the MFO enzyme system responsible for benzene metabolism. Although its direct effects on benzene metabolism have not been investigated, Pb administered ip or iv depresses heme synthesis and cytochrome P-450 synthesis and alters cytochrome P-450 related enzyme activities (Alvares et al., 1972, 1976; Scoppa et al., 1973; Penning and Scoppa, 1977).

We observed a significant increase in the rate at which benzene was converted to phenol in liver supernatant enzyme preparations from animals treated with Pb. Alvares et al. (1976) investigated the effects of oral Pb on cytochrome P-450 mediated enzyme activities. These authors fed weanling rats a 2% lead acetate diet for 12 wk, sacrificed them after 3, 6, and 12 wk, and examined activities of liver microsomal preparations. They observed no significant changes in microsomal protein concentrations, cytochrome P-450 levels, or cytochrome P-450 mediated activities. Although the MFO system metabolizes and detoxifies many foreign compounds, the effects of Pb that we observed may be specific to benzene or similar compounds. Also, the difference between these results could be due to our method of enzyme preparation. The supernatant fraction we used contained all cellular components except nuclei and mitochrondria. The microsomal preparations of Alvares et al. had the cellular membrane fraction that contained the MFO system responsible for benzene hydroxylation. It is possible that an unidentified component present in our supernatant but absent in microsomal preparations was responsible for the increased activity of liver preparations from Pb-treated animals.

The purpose of our 24-wk study was to test the effects of long-term Pb ingestion on the *in vivo* metabolism of benzene to phenol. Since Pb interferes with heme production, and a large fraction of the heme synthesized by the liver is incorporated into the cytochrome P-450 enzyme (Estabrook et al., 1970), we examined the effect of Pb on the *in vivo* metabolism of benzene to phenol by the intact cytochrome P-450 enzyme system.

We found that chronic administration of Pb had no effect on urinary phenol excretion after an ip injection of benzene. This is in good agreement with other recent results. No differences were reported in the abilities of normal and Pb-poisoned children to metabolize two drugs known to be dependent on the MFO system (Alvares et al., 1975). In addition, human adults exposed for 3 mo to Pb had clinical signs of Pb poisoning but normal half-lives for drugs metabolized by the liver microsomal system (Fischbein et al., 1977).

Our study indicated that the effect of Pb on heme-dependent cytochrome P-450 enzymes *in vitro* was not enough to significantly alter heme-dependent activity *in vivo*. The MFO system appeared to compensate for any effects of chronic Pb administration. However, an effect of Pb on

benzene metabolism might be observed in the production of other benzene metabolites, such as hydroxyquinone or catechol, which were not measured here.

In general, the urinary phenol levels before benzene administration were much lower than previously observed. Control, free, and total phenol levels were approximately 1% of those reported earlier (Cornish and Ryan, 1965). In the first 24 h after benzene injection, free and total phenol increased to 24 μg and 30 mg, respectively. Cornish and Ryan (1965) reported a higher free phenol level (3 mg) but a lower total phenol level (17 mg) 24 h after injection. The difference between our results is probably due to the fact that the gas chromatography method we employed for phenol detection is more sensitive than the Folin-Ciocalteau spectrophotometric method employed by Cornish and Ryan.

Urinary phenol can account for 9–40% of a benzene dose (Parke and Williams, 1953; Cornish and Ryan, 1965; Dutkiewicz, 1971; Timbrell and Mitchell, 1977). We found that phenol excretion was complete 48 h after benzene administration and a total of 36% of the benzene dose was excreted as phenol.

Pb accumulated in bones and soft tissues of animals after 15 and 24 wk. The Pb concentration in bones was significantly elevated only in animals that received the 352 ppm Pb solution. This was detected after 6 wk of Pb treatment and continued throughout the 24-wk study. Pb concentrations in liver tissue significantly increased in each study with time and treatment level. Significant amounts of Pb accumulated in liver tissue only in samples from animals in the 352 ppm Pb group and only after 9 wk of Pb dosing.

Although Pb accumulated in bone and liver tissues with time of treatment, enzyme activity was increased most markedly after 6 wk. The absolute amount of Pb administered did not appear to be as important as the 6-wk latency period for increased enzyme activity. Furthermore, a scatter plot of liver Pb concentration and enzyme activity showed no significant relation. This suggested that sensitivity to *in vivo* Pb administration is apparent after 6 wk but does not increase with continued Pb accumulation. The results of both studies indicate that further investigations are needed, especially on the effects of Pb on benzene metabolism in bone marrow. Since bone marrow tissue is most sensitive to the neoplastic effects of benzene, it would be valuable to investigate Pb-benzene interactions that could occur there.

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